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Cyclic AMP Increases Cell Surface Expression of Functional Na,K-ATPase Units in Mammalian Cortical Collecting Duct Principal Cells

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Cyclic AMP (cAMP) stimulates the transport of Na^+ and Na,K-ATPase activity in the renal cortical collecting duct (CCD). The aim of this study was to investigate the mechanism whereby cAMP stimulates the Na,K-ATPase activity in microdissected rat CCDs and cultured mouse mpkCCD$_{c14}$ collecting duct cells. db-cAMP (10^{-3} M) stimulated by 2-fold the activity of Na,K-ATPase from rat CCDs as well as the ouabain-sensitive component of $^{86}Rb^+$ uptake by rat CCDs (1.7-fold) and cultured mouse CCD cells (1.5-fold). Pretreatment of rat CCDs with saponin increased the total Na,K-ATPase activity without further stimulation by db-cAMP. Western blotting performed after a biotinylation procedure revealed that db-cAMP increased the amount of Na,K-ATPase at the cell surface in both intact rat CCDs (1.7-fold) and cultured cells (1.3-fold), and that this increase was not related to changes in Na,K-ATPase internalization. Brefeldin A and low temperature (20°C) prevented both the db-cAMP-dependent increase in cell surface expression and activity of Na,K-ATPase in both intact rat CCDs and cultured cells. Pretreatment with the intracellular Ca^{2+} chelator bis-(*o*-aminophenoxy)-*N*,*N*,*N*′,*N*′-tetraacetic acid also blunted the increment in cell surface expression and activity of Na,K-ATPase caused by db-cAMP. In conclusion, these results strongly suggest that the cAMP-dependent stimulation of Na,K-ATPase activity in CCD results from the translocation of active pump units from an intracellular compartment to the plasma membrane.

INTRODUCTION

Na,K-ATPase located at the basolateral membrane of kidney epithelial cells provides the driving force for active Na^+ and K^+ transport, and for secondary active transport of other solutes. Accordingly, the activity of renal tubule Na,K-ATPase is tightly regulated (Féraille and Doucet, 2000). Long-term regulation of Na,K-ATPase relies mainly on alterations of the expression of its subunits, whereas short-term control is mediated by phosphorylation and/or redistribution between the cell surface and intracellular compartments.

Barlet-Bas *et al.* (1990) and Blot-Chabaud *et al.* (1990) first reported that an acute increase in intracellular concentration of Na^+ rapidly stimulates the V_{max} of Na,K-ATPase activity and increases the number of active pump units, taken as the specific [^{3}H]ouabain binding, in the mammalian cortical collecting duct (CCD). These studies strongly suggested that an inactive pool of Na,K-ATPase units can be rapidly activated under particular circumstances. However, whether the increased number of active Na,K-ATPase units results from the translocation of pumps from an intracellular compartment to the plasma membrane and/or the activation of latent pump units already located at the plasma membrane still remains to be determined.

We have recently shown that the rapid protein kinase A (PKA)-dependent stimulation of Na,K-ATPase activity in

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proximal convoluted tubule (PCT) cells relies on an increase in cell surface expression of Na,K-ATPase units (Carranza *et al.*, 1998). However, this study did not permit to conclude whether the activation of PKA decreased the rate of internalization of Na,K-ATPase or increased that of delivery to the plasma membrane.

In CCD, PKA-dependent stimulation of Na,K-ATPase accounts in part for the stimulatory effect of vasopressin on sodium reabsorption (Blot-Chabaud *et al.*, 1990). The aim of this study was to elucidate whether the cAMP-dependent stimulation of Na,K-ATPase activity 1) is linked to an increase in Na,K-ATPase at the cell membrane, and 2) whether such increase results from alterations in the delivery to and/or the withdrawal of Na,K-ATPase from the plasma membrane in mammalian CCD. To answer these questions, experiments were performed on intact microdissected rat CCDs and in the immortalized mouse collecting duct principal cell line mpkCCD$_{c14}$ (Bens *et al.*, 1999) to validate this cell system model for future investigation on the intracellular mechanisms underlying the regulatory processes of Na,K-ATPase.

MATERIALS AND METHODS

Isolated Rat Kidney Tubules. Male Wistar rats (150–200 g of body weight; Center Médical Universitaire, Genève, Switzerland, or Elevage Janvier, Le Genest-St-Isle, France) were anesthetized with pentobarbital (5 mg/100 g of body weight i.p.) and the left kidney was perfused with incubation solution (120 mM NaCl, 5 mM RbCl, 4 mM $NaHCO_3$, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.2 mM NaH_2PO_4, 0.15 mM Na_2HPO_4, 5 mM glucose, 10 mM lactate, 1 mM pyruvate, 4 mM essential and nonessential amino acids, 0.03 mM vitamins, 20 mM HEPES, 0.1% bovine serum albumin [BSA], pH 7.45) containing 0.44% (wt/vol) collagenase (CLSII, 0.75–0.87 U/mg; Serva, Heidelberg, Germany). Afterward, the kidney was removed, sliced into small pyramids, and incubated 20 min at 30°C in oxygenated (95% O_2 and 5% CO_2) incubation solution containing 0.08% (wt/vol) collagenase. Single CCDs were isolated by microdissection in ice-cold oxygenated incubation solution containing aprotinin (10 mTIU/ml) and leupeptin (20 mg/ml) to preserve the integrity of tubules. The length of tubular segments, which served as reference for Na,K-ATPase activities and for Western blot analysis, was determined after photography of microdissected CCDs. The PCT-enriched suspensions were obtained as described previously (Carranza *et al.*, 1996) by mechanical dissociation of the kidney cortex through 150- and 100-μm pore size nylon filters.

Cell Culture. The mpkCCD$_{c14}$ cell line, a mouse cortical collecting duct principal cell line exhibiting mineralocorticoid-dependent sodium transport (Bens *et al.*, 1999), was cultured in modified DM medium (DMEM: Ham's F12, 1:1 vol/vol, 60 nM sodium selenate, 5 μg/ml transferrin, 2 mM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 20 mM D-glucose, 2% decomplemented fetal calf serum [FCS], and 20 mM HEPES, pH 7.4) at 37°C in 5% CO_2/95% air atmosphere. The medium was changed every 2 d and the experiments were performed on passages 18–40.

Measurement of the Ouabain-sensitive ^{86}Rb Uptake. The transport activity of Na,K-ATPase was measured by the ouabain-sensitive $^{86}Rb^+$ uptake under conditions of initial rate, as previously described (Cheval and Doucet, 1990; Féraille *et al.*, 1992). Before the $^{86}Rb^+$ uptake assay, isolated rat CCDs were submitted to various treatments in the presence or in the absence of ouabain (2.5 mM). The ouabain-sensitive $^{86}Rb^+$ uptake was determined as the difference between the mean values measured in 5–7 replicate samples incubated without and with 2.5 mM ouabain, respectively. $^{86}Rb^+$ uptake was expressed as picomoles of Rb^+ per millimeter of tubule per minute.

Confluent mpkCCD$_{c14}$ cells (7 d) grown on polycarbonate semipermeable transwell filters (12 mm in diameter, 0.4-μm pore size; Costar, Cambridge, MA) were preincubated in FCS-free culture medium with or without ouabain (2 mM) for 60 min at 37°C. Cells were then incubated in the absence or presence of drugs added to the apical and basolateral side of the filters. The transport activity of Na,K-ATPase was determined in quadruplicate samples after the addition of 50 μl of medium containing tracer amounts of ^{86}RbCl (100 nCi/sample; Amersham, United Kingdom) for 3 min. Incubation was stopped by cooling on ice, rapid aspiration of the incubation medium in the two compartments, and three washes with an ice-cold washing solution containing 150 mM choline-chloride, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 2 mM $BaCl_2$, and 5 mM HEPES, pH 7.4. Cells were lysed in 750 μl of Triton X-100 1% (wt/vol) and the radioactivity was measured by liquid scintillation on 400-μl samples. Protein content was determined in parallel by using the bicinchoninic acid assay (Pierce, Rockford, IL). The ouabain-sensitive $^{86}Rb^+$ uptake was calculated as the difference between the mean values measured in quadruplicate samples incubated with or without 2 mM ouabain and was expressed as picomoles of Rb per microgram of protein per minute. Preliminary experiments have shown that the rate of $^{86}Rb^+$ uptake was linear for at least 5 min at 37°C and that ouabain-sensitive $^{86}Rb^+$ uptake accounted for approximately two-thirds of the total $^{86}Rb^+$ uptake.

Measurement of Na,K-ATPase Activity. The hydrolytic activity of Na,K-ATPase was determined under V_{max} conditions by the release of ^{32}Pi from [γ-^{32}P]ATP on permeabilized rat isolated CDDs, as previously described (Doucet *et al.*, 1979; Deschênes and Doucet, 2000). CCDs were incubated in the absence or presence of db-cAMP for 15 min at 37°C. After incubation, pools of 4–6 CCDs were transferred in 0.5 μl of incubation solution into the BSA-coated wells of a flat-bottomed plastic microplate and were photographed for determination of their length. In most experiments, cell membrane of CCDs was permeabilized by freeze/thawing in an hypoosmotic medium, as follows: 2 μl of 10 mM Tris-HCl (pH 7.4) was added to each well, and the tubules were submitted to a freeze/thawing step on dry ice. In some experiments, permeabilization was performed using saponin (see RESULTS). Whatever the permeabilization procedure, the microplate was incubated for 15 min at 37°C after addition of 10 μl of ATPase assay solution to each well. The total ATPase activity was measured in a solution containing 100 mM NaCl, 50 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 100 mM Tris-HCl, 10 mM Na_2ATP, trace amounts (5 nCi/μl) of [γ-^{32}P]ATP [2–10 Ci/mmol; New England Nuclear, Boston, MA] and adjusted at pH 7.4. For Na^+,K^+-independent ATPase activity measurements, NaCl and KCl were replaced by 150 mM choline chloride and 2 mM ouabain was added. The reaction was stopped by addition of 200 μl of an ice-cold 10% (wt/vol) suspension of activated charcoal. The microplate was centrifuged at 4°C and 50 ml of each supernatant was transferred into 6-ml vials and radioactivity was determined by liquid scintillation. The Na,K-ATPase activity was taken as the difference between the mean total and Na^+,K^+-independent ATPase activities measured in quadruplicate samples. Results were expressed as picomoles of ATP per millimeter of tubule per hour.

Measurement of Total and Cell Surface Na,K-ATPase. Pools of 100 microdissected CCDs or suspensions of cortical tubules were incubated in BSA-free incubation solution (120 mM NaCl, 5 mM RbCl, 4 mM $NaHCO_3$, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.2 mM NaH_2PO_4, 0.15 mM Na_2HPO_4, 5 mM glucose, 10 mM lactate, 1 mM pyruvate, 4 mM essential and nonessential amino acids, 0.03 mM vitamins, and 20 mM HEPES, pH 7.45) with or without drugs.

For measurement of total tubular content of Na,K-ATPase, CCDs were pelleted and lysed in homogenization buffer (HB) (20 mM Tris-HCl pH 7.4, 2 mM EDTA, 2 mM EGTA, 20 μg/ml leupeptin, 10

mTIU/ml aprotinin, 30 mM NaF, 30 mM Na pyrophosphate, and containing 1 mM phenylmethylsulfonyl fluoride, 1 mM AEBSF, 0.1% [wt/vol] SDS, and 1% (vol/vol) Triton X-100). Lysed samples were resuspended in Laemmli's buffer (Laemmli, 1970) and analyzed by SDS-PAGE. After electrophoresis on 7% polyacrylamide gels, proteins were electrotransferred on polyvinylidine difluoride membranes (Immobilon-P; Millipore, Waters, MA), and incubated overnight with polyclonal antibody (dilution 1/2500) raised against the α-subunit of Na,K-ATPase (Carranza *et al.*, 1996) in Tris-buffered saline (TBS) NP-40 (150 mM NaCl, 50 mM Tris, 0.2% NP-40, pH 7.4) with 5% (wt/vol) dried nonfat milk. After washing in TBS-NP-40, membranes were incubated with an anti-rabbit IgG antibody (dilution 1/10 000) coupled to horseradish peroxidase (Transduction Laboratories, Lexington, KY) in TBS NP-40. The antigen–antibody complexes were detected by chemiluminescence with the Super Signal Substrate method (Pierce) according to the manufacturer's instructions.

Measurement of cell surface Na,K-ATPase was performed on cortical tubule suspensions or isolated rat CCDs. Samples were biotinylated in BSA-free incubation solution containing 1.5 mg/ml EZ-Link sulfossuccinimidobiotin (sulfo NHS-S-S-biotin; Pierce) for 1 h at 4°C on a roller system. After labeling, tubules were pelleted and placed in an incubation solution containing 0.1% BSA (wt/vol) for 30 min at 4°C to quench the free unreacted sulfo NHS-S-S-biotin. After centrifugation, tubules were lysed in HB buffer, and biotinylated proteins were precipitated in the presence of streptavidin-agarose beads (Immunopure immobilized streptavidin; Pierce) diluted in an antiprotease-containing buffer solution (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, 20 μg/ml leupeptin, 10 mTIU/ml aprotinin) and incubated overnight at 4°C. The beads were then washed twice with rinsing solution A (50 mM Tris-HCl pH 7.4, 5 mM EDTA, 50 mM NaCl), twice with rinsing solution B (20 mM Tris-HCl pH 7.4, 500 mM NaCl) and once with 10 mM Tris-HCl, pH 7.4. After resuspension in Laemmli's buffer, samples were processed for SDS-PAGE and Western blotting as described above. In each experiment carried out on microdissected CCDs from different rats, the amounts of proteins loaded to each lane of the electrophoresis gel corresponded to the same initial length of isolated CCDs (±5%). The chemiluminescence of each lane was quantified by densitometry (arbitrary units), and expressed in each experiment as percentage of the control lane (no cAMP treatment). Results were expressed as means ± SE from several animals.

Confluent mpkCCD$_{c14}$ cells (day 7 after seeding) grown on semi-permeable polycarbonate transwell filters (12 mm in diameter, 0.4-μm pore size; Costar) were preincubated in FCS-free medium with or without drugs for 60 min at 37°C. Cells were incubated in the absence or presence of db-cAMP added to the apical and basolateral side of the filter. Cell surface proteins were then biotinylated by adding PBS-CM (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 6.4 mM Na_2HPO_4, 0.1 mM $CaCl_2$, and 1 mM $MgCl_2$) containing 1.5 mg/ml sulfo NHS-S-S-biotin to the apical and basolateral side of the filter for 1 h at 4°C. The free unreacted sulfo NHS-S-S-biotin was quenched by incubation with PBS-CM containing 0.1% BSA (wt/vol) for 30 min at 4°C. Cells were then lysed in HB as described above and the protein content was determined using the bicinchoninic protein assay (Pierce). Equal amounts of proteins were precipitated by streptavidin-agarose beads and analyzed by Western blotting as described above.

Measurement of Na,K-ATPase Internalization. Isolated rat CCDs were first biotinylated (see above) and then incubated in the absence or presence of drugs. Tubules were rinsed four times in a reducing solution (50 nM Tris pH 8.6, 100 mM NaCl, 25 mM 2-(*N*-morpholino)ethanesulfonic acid [MES]-Na, and 25 mM dithiothreitol [DTT]) during 10 min at 4°C to allow the debiotinylation of proteins remaining at the cell surface. After two rinses in PBS, tubules were lysed in HB and incubated in the presence of streptavidin beads. Internalized Na,K-pumps protected from reducing agents were detected by Western blotting as described above.

Adenylyl Cyclase Assay. Adenylyl cyclase activity was determined on single CCDs by the rate of conversion of [α-^{32}P]ATP into [^{32}P]cAMP under basal and vasopressin-stimulated conditions (10^{-8} M) according to the method described Imbert *et al.* (1975). Briefly, once microdissected, CCDs were incubated with or without vasopressin and then permeabilized by freeze/thawing in an hypoosmotic medium in the absence or presence of saponin (0.5 mg $\cdot$ ml^{-1}). CCDs were then incubated at 30°C for 30 min with [α-^{32}P]ATP in the presence of an ATP-regenerating system (phosphocreatine and creatine kinase) and after incubation, the [^{32}P]cAMP was separated from the other ^{32}P nucleotides by double column filtration (Dowex and alumine) procedure. The yield of the whole separation procedure was evaluated in each sample by the recovery of [^{3}H]cAMP added to each sample at the end of the incubation. Measurements were performed on 5–6 replicates and results were expressed as femtomoles of cAMP per millimeter of tubule per 30 min.

Statistics. Statistical analysis of Rb uptakes and Na,K-ATPase activities were done by unpaired Student *t* test or by analysis of variance for comparison of two or more than two groups, respectively. Statistical analysis of Na,K-ATPase α-subunit immunoreactivity was done using the Mann-Whitney *U* test or the Kruskal-Wallis test for comparison of two or more than two groups, respectively. Results are expressed as means ± SE from (n) independent experiments. Each experiment was performed with tubules from one animal or with cells from one passage. A p value less <0.05 was considered significant.

RESULTS

db-cAMP Stimulated the Activity of Na,K-ATPase in Isolated Rat CCDs and in Cultured mpkCCD$_{c14}$ Cells

The effect of db-cAMP (*N*, 2′-*O*-dibutyryladenosine, 3′:5′-cyclic monophosphate; Sigma, St. Louis, MO), a cell-permeant analog of cAMP, on the transport and hydrolytic activities of Na,K-ATPase was determined in rat CCDs and in mpkCCD$_{c14}$ cells. Incubation of CCDs with 10^{-3} M db-cAMP for 15 min at 37°C stimulated ouabain-sensitive $^{86}Rb^+$ uptake by 73% (control, 8.5 ± 1.0; db-cAMP, 14.4 ± 2.9 pmol Rb $\cdot$ mm^{-1} $\cdot$ min^{-1}; n = 5; $p < 0.05$) and Na,K-ATPase activity by 78% (Figure 1, A and B) (control, 337 ± 54; db-cAMP, 606 ± 67 pmol ATP $\cdot$ mm^{-1} $\cdot$ h^{-1}; n = 5; $p < 0.05$). The transport activity of Na,K-ATPase was also increased by 49% in mpkCCD$_{c14}$ cells incubated with 10^{-3} M db-cAMP for 30 min at 37°C (Figure 1C) (control, 11.8 ± 1.8; db-cAMP, 17.2 ± 2.4 pmol Rb $\cdot$ μg protein^{-1} $\cdot$ min^{-1}; n = 6; $p < 0.05$). Similar results were obtained in mpkCCD$_{c14}$ cells incubated with 10^{-5} M forskolin, which increases the endogenous cAMP level through direct activation of adenylyl cyclase (data not shown). Thus, db-cAMP, mimicking an increase in intracellular cAMP level, stimulated Na,K-ATPase in CCD cells.

Permeabilization of Rat CCDs with Saponin Mimicked Stimulation of Na,K-ATPase by cAMP

In the previous experimental series, Na,K-ATPase hydrolytic activity was determined in CCDs permeabilized by the classical hypoosmotic and freeze/thawing method (Doucet *et al.*, 1979). Under such conditions, only cell plasma membrane-associated ATPase activities but not the intracellular organelle's membrane-associated ATPase activities are measured (Khadouri *et al.*, 1991; Chibalin *et al.*, 1999). Because

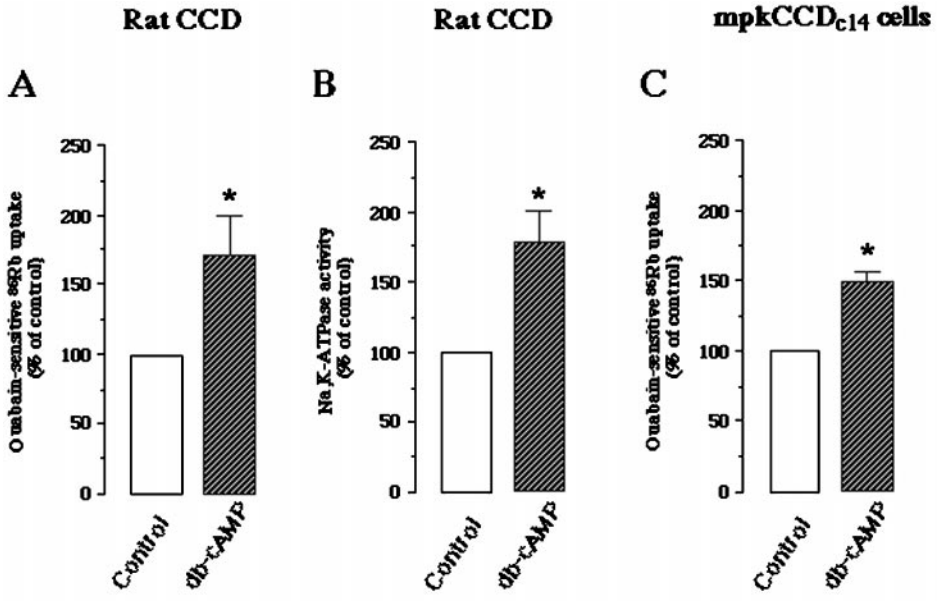

Figure 1. Effect of db-cAMP on Na,K-ATPase activity in rat CCDs and in mpkCCD$_{c14}$ cells. (A and B) Effect of db-cAMP on the transport and hydrolytic activities of Na,K-ATPase in rat CCDs. The ouabain-sensitive ^{86}Rb uptake (A) and the hydrolytic activity of Na,K-ATPase (B) were determined in microdissected rat CCDs preincubated in the absence or presence of 10^{-3} M db-cAMP for 15 min at 37°C. Values (means ± SE from 5 independent experiments) are expressed as percentage of controls (^{86}Rb uptake: 8.5 ± 1.0 pmol $Rb^+ \cdot mm^{-1} \cdot min^{-1}$; Na,K-ATPase: 337 ± 54 pmol $ATP \cdot mm^{-1} \cdot h^{-1}$). *, $p < 0.05$ versus control values. (C) Effect of db-cAMP on the transport activity of Na,K-ATPase in mpkCCD$_{c14}$ cells. The rate of ouabain-sensitive ^{86}Rb uptake was determined in mpkCCD$_{c14}$ cells preincubated in the absence or presence of 10^{-3} M db-cAMP for 30 min at 37°C. Values (means ± SE from 6 independent experiments) are expressed as percentage of controls (11.8 ± 1.8 pmol $Rb^+ \cdot \mu g$ $protein^{-1} \cdot min^{-1}$). *, $p < 0.05$ versus control values.

treatment with detergent was reported to unmask a latent intracellular pool of Na,K-ATPase activity in proximal tubule cells (Chibalin *et al.*, 1999), we used saponin to permeabilize both plasma and intracellular organelle membranes. Figure 2 shows that permeabilization of rat CCDs with saponin (saponin 0.5 mg · ml^{-1} for 10 min at room temperature) increased Na,K-ATPase by 90% compared with CCDs permeabilized by the freeze/thawing method (freeze/thawing, 486 ± 50; saponin, 920 ± 87 pmol $ATP \cdot mm^{-1} \cdot h^{-1}$; n = 5; $p < 0.005$). Furthermore, preincubation with db-cAMP (10^{-3} M for 30 min at 37°C) no further increased Na,K-ATPase activity measured after saponin permeabilization (control, 920 ± 87; db-cAMP, 966 ± 127 pmol $ATP \cdot mm^{-1} \cdot h^{-1}$; n = 5; NS) in contrast to that observed under freeze/thawing permeabilization (control, 486 ± 50; db-cAMP, 887 ± 113 pmol $ATP \cdot mm^{-1} \cdot h^{-1}$; n = 5; $p < 0.025$), suggesting that cAMP and saponin revealed the activity of the same pool of Na,K-ATPase. On the other hand, saponin permeabilization of isolated CCDs did not alter the basal and the vasopressin-stimulated (10^{-8} M) adenylyl cyclase activity (freeze/thawing basal, 100 ± 6; saponin basal, 96 ± 4; freeze/thawing + vasopressin, 978 ± 60; saponin + vasopressin, 949 ± 80 fmol · mm^{-1} · 30 min^{-1}; n = 6).

These results suggested thus the presence of an intracellular pool of Na,K-ATPase (undetectable without permeabilization of intracellular organelles membranes) and showed that cAMP increases the cell surface expression of this pool of Na,K-ATPase. Therefore, the quantification of total and cell surface Na,K-ATPase by Western blotting was performed to confirm these observations.

db-cAMP Increased the Cell Surface Amount of Na,K-ATPase in Rat CCDs and in Cultured mpkCCD$_{c14}$ Cells

Figure 3, A–D, depicts the effect of db-cAMP on the total cell and cell surface amounts of Na,K-ATPase in rat CCDs, estimated by Western blotting. Preincubation with db-cAMP (10^{-3} M for 15 min at 37°C) did not change the total cellular content of Na,K-ATPase (Figure 3, A and B) (as percentage of control: db-cAMP, 101 ± 6%; n = 4; NS), whereas it markedly increased the amount of Na,K-ATPase accessible

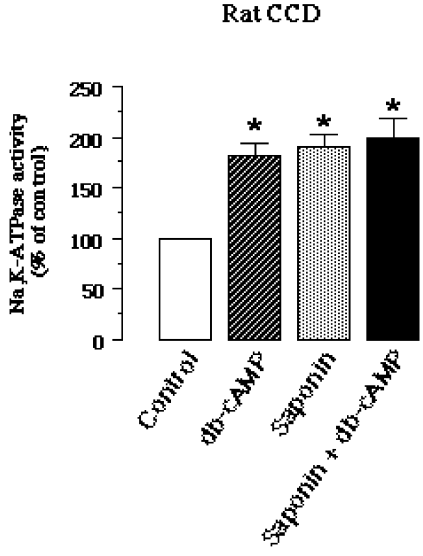

Figure 2. Saponin unmasked db-cAMP-stimulated Na,K-ATPase hydrolytic activity in rat CCD. Microdissected CCDs were preincubated in the absence or presence of 10^{-3} M db-cAMP for 30 min at 37°C. The hydrolytic activity of Na,K-ATPase was measured after permeabilization of cell membranes by using the freeze/thawing procedure or by incubating CCDs with 0.5 mg · ml^{-1} saponin for 10 min at room temperature. Values (means ± SE from 5 independent experiments) are expressed as percentage of controls (486 ± 50 pmol $ATP \cdot mm^{-1} \cdot h^{-1}$). *, $p < 0.05$ versus control values.

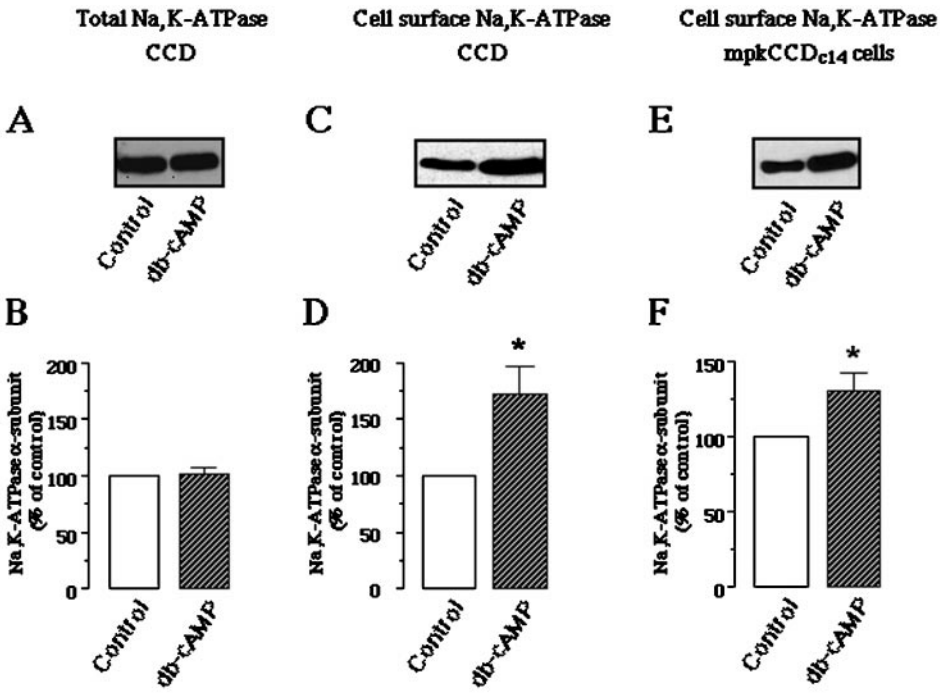

Figure 3. Effect of db-cAMP on total and cell surface amount of Na,K-ATPase in rat CCDs and in mpkCCD$_{c14}$ cells. (A and B) Total amount of Na,K-ATPase in control and db-cAMP treated rat CCDs. Same amounts of microdissected rat CCDs were incubated in the absence or presence of 10^{-3} M db-cAMP for 15 min at 37°C. CCDs were then lysed and the Na,K-ATPase α-subunit was detected by Western blotting using a specific polyclonal antibody. (A) Representative immunoblot. (B) Bars represent the densitometric quantification (means ± SE) from four different experiments. Results are expressed as percentage of the optical density values from untreated samples (control). (C–F) Cell surface amount of Na,K-ATPase in control and db-cAMP-treated rat CCDs and mpkCCD$_{c14}$ cells. Microdissected rat CCDs or mpkCCD$_{c14}$ cells were incubated in the absence or presence of 10^{-3} M db-cAMP for 15 min (CCDs) or 30 min (mpkCCD$_{c14}$) at 37°C. After biotinylation, samples were lysed and labeled proteins were precipitated by streptavidin-agarose beads. The Na,K-ATPase α-subunit was detected by Western blotting using a specific polyclonal antibody. (C and E) Representative immunoblots. (D and F) Bars represent the densitometric quantification (means ± SE) from 12 independent experiments. Results are expressed as percentage of the optical density values from untreated samples (control); *, $p < 0.05$ versus control values.

to biotin at the cell surface (Figure 3, C and D) (as percentage of control: db-cAMP, 173 ± 24%; n = 12; $p < 0.05$).

As shown in Figure 3, E–F, db-cAMP (10^{-3} M for 30 min at 37°C) also increased the amount of Na,K-ATPase at the surface of mpkCCD$_{c14}$ cells (as percentage of control: db-cAMP, 130 ± 12%; n = 12; $p < 0.05$). Figure 4 depicts the time course of db-cAMP effect on the cell surface expression of Na,K-ATPase. In mpkCCD$_{c14}$ cells, the increased amount of cell surface Na,K-ATPase caused by db-cAMP was observed after 10 min (10^{-3} M at 37°C) and sustained for at least 30 min. These results indicated that db-cAMP did not change the total cellular content of Na,K-ATPase but induced an increase in the fraction of Na,K-ATPase present at the cell surface of CCD principal cells.

db-cAMP Did Not Alter Internalization of Na,K-ATPase in Rat CCDs

A biotinylation-debiotinylation procedure was applied to determine whether db-cAMP decreased the internalization of cell surface Na,K-ATPase in rat CCDs. The efficiency of the biotinylation-debiotinylation procedure to analyze the internalization of Na,K-ATPase was first assessed on proximal tubule suspensions (Figure 5, A and B) where dopamine has been shown to induce Na,K-ATPase internalization (Chibalin *et al.*, 1997). When biotinylated PCTs were incubated at 4°C in the presence of a reducing buffer containing DTT and Mes-Na, the biotinylated Na,K-ATPase α-subunit was no longer detected by Western blotting in whole cell lysates (Figure 5A). This control experiment dem-

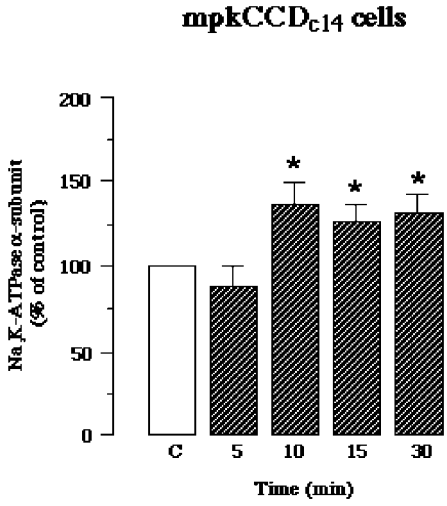

Figure 4. Time course of the effect of db-cAMP on cell surface Na,K-ATPase in mpkCCD$_{c14}$ cells. Cells were incubated in the absence (C) or presence of 10^{-3} M db-cAMP for 5, 10, 15, or 30 min at 37°C. After biotinylation, cells were lysed and labeled proteins were precipitated by streptavidin-agarose beads. The Na,K-ATPase α-subunit was detected by Western blotting as described in Figure 3. Bars represent the densitometric quantification (means ± SE) from six independent experiments. Results are expressed as percentage of the optical density values from untreated samples (C); *, $p < 0.05$ versus control values.

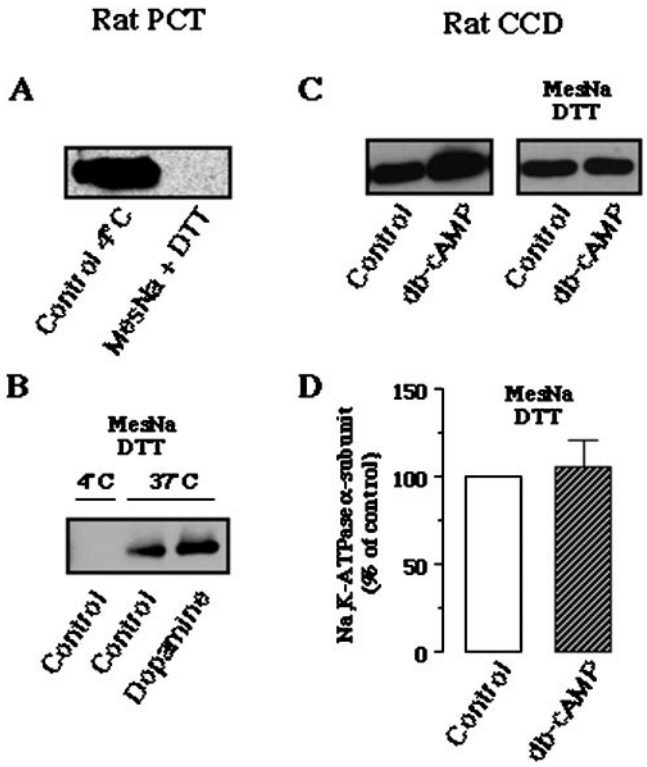

Figure 5. Internalization of Na,K-ATPase: effect of db-cAMP in rat CCDs. (A and B) Validation of the biotinylation/debiotinylation procedure on rat PCT suspensions. (A) Rat PCTs were first biotinylated and then either maintained at 4°C or immediately washed four times at 4°C in reducing buffer (Mes-Na, DTT) to allow debiotinylation of cell surface proteins. After cell lysis and streptavidin agarose-beads precipitation, the Na,K-ATPase α-subunit was detected by Western blotting as described in Figure 3. (B) After biotinylation, rat PCTs were debiotinylated at 4°C by the Mes-Na-DTT buffer either immediately or after incubation for 30 min at 37°C in the absence or presence of 10^{-6} M dopamine. After cell lysis, labeled proteins were precipitated by streptavidin agarose-beads, and Na,K-ATPase α-subunit was detected by Western blotting as described in Figure 3. (C and D) Effect of db-cAMP on the internalization of Na,K-ATPase in rat CCDs. (C, left) Microdissected rat CCDs were incubated without or with 10^{-3} M db-cAMP for 15 min at 37°C. After biotinylation, CCDs were lysed, labeled proteins were precipitated by streptavidin-agarose beads, and Na,K-ATPase α-subunit was detected by Western blotting. (C, right) Microdissected rat CCDs were biotinylated and incubated in absence or presence of 10^{-3} M db-cAMP for 15 min at 37°C. Tubules were then debiotinylated by washes at with 4°C Mes-Na-DTT buffer, lysed, and labeled proteins were precipitated by streptavidin agarose-beads and the Na,K-ATPase α-subunit was analyzed by Western blotting. (D) Bars represent the densitometric quantification (means ± SE) from nine experiments such as those shown in C (right). Results are expressed as percentage of the optical density values from untreated samples (control).

onstrated that incubation at 4°C fully inhibited Na,K-ATPase internalization and that the incubation in the DTT-Mes-Na buffer reduced all the disulfide bridges of the biotin label. Preincubation of biotinylated PCTs with dopamine (10^{-6} M at 37°C for 30 min) before debiotinylation at 4°C in the DTT-Mes-Na buffer increased the amount of streptavidin-precipitable Na,K-ATPase in cell lysates (Figure 5B). Therefore, these results confirmed that dopamine protected more Na^+ pumps from debiotinylation by the reducing buffer, as a reflection of increased internalized Na,K-ATPase. This methodology thus appeared suitable to study the possible effect of db-cAMP on Na,K-ATPase internalization in microdissected rat CCDs.

In experiments in which the stimulatory effect of db-cAMP (10^{-3} M, 15 min at 37°C) on the amount of cell membrane Na,K-ATPase was assessed in rat CCDs (Figure 5C, left), the internalization of Na,K-ATPase appeared to be unmodified, because the amount of streptavidin-precipitable Na,K-ATPase measured after debiotinylation was unchanged following db-cAMP stimulation (Figure 5C, right, and D). The observed increase in cell surface Na,K-ATPase induced by db-cAMP that was not associated with changes in total cellular amount of Na,K-ATPase and Na,K-ATPase internalization suggested that db-cAMP stimulated the insertion of Na,K-ATPase units from an intracellular pool to the plasma membrane.

Brefeldin A Prevented db-cAMP–induced Cell Surface Expression and Activity of Na,K-ATPase

To assess whether the increase in Na,K-ATPase cell surface expression caused by db-cAMP resulted from a vesicular trafficking-dependent process, experiments were performed on rat CCDs and cultured mpkCCD$_{c14}$ cells pretreated with brefeldin A (Bref A), an agent known to disrupt the ARF1-dependent vesicular traffic (Figure 6). As controls, indirect immunofluorescence experiments using a monoclonal antibody against βCOP have shown that 20 μg/ml Bref A (1 h at 30°C) induced disruption of the Golgi apparatus in mpkCCD$_{c14}$ cells (data not shown). Rat CCDs and mpkCCD$_{c14}$ cells were pretreated with 20 μg/ml Bref A for 1 h at 30°C and then incubated at 37°C without or with 10^{-3} M db-cAMP for 15 min. Figure 6, D and E, shows that Bref A alone did not modify significantly Na,K-ATPase cell surface expression in mpkCCD$_{c14}$ cells (as percentage of control: Bref A, 113 ± 8%). In contrast, Bref A abolished db-cAMP-induced increase in Na,K-ATPase cell surface expression in rat CCDs (Figure 6, A and B) and in mpkCCD$_{c14}$ cells (Figure 6, D and E) (as percentage of Bref A: rat CCD, Bref A + db-cAMP, 108 ± 4%; n = 4; NS; and mpkCCD$_{c14}$ cells, Bref A + db-cAMP, 80 ± 13%; n = 4; NS). Brefeldin A also abolished the stimulation of Na,K-ATPase activity induced by db-cAMP in CCDs (Figure 6C) (Bref A, 403 ± 84; Bref A + db-cAMP, 433 ± 119 pmol ATP · mm^{-1} · h^{-1}; n = 7; NS). Thus, db-cAMP increased the cell surface expression and activity of Na,K-ATPase through a brefeldin A-dependent process in CCD cells.

Temperature- and Ca^{2+}-Dependencies of db-cAMP–induced Increase in Cell Surface Expression of Na,K-ATPase

The role of temperature and of intracellular Ca^{2+}, two parameters that interfere with vesicular trafficking process, were evaluated on cAMP-induced cell surface expression of Na,K-ATPase in rat CCDs and mpkCCD$_{c14}$ cells incubated at 20°C or at 37°C with the Ca^{2+} chelator BAPTA-AM (glycine, *N,N′*-[1,2-ethanediylbis(oxy-2,1-phenylene)bis[*N*-[2-[(acetyloxy)methoxy]-2-oxoethyl]], bis[(acetyloxy)methyl]

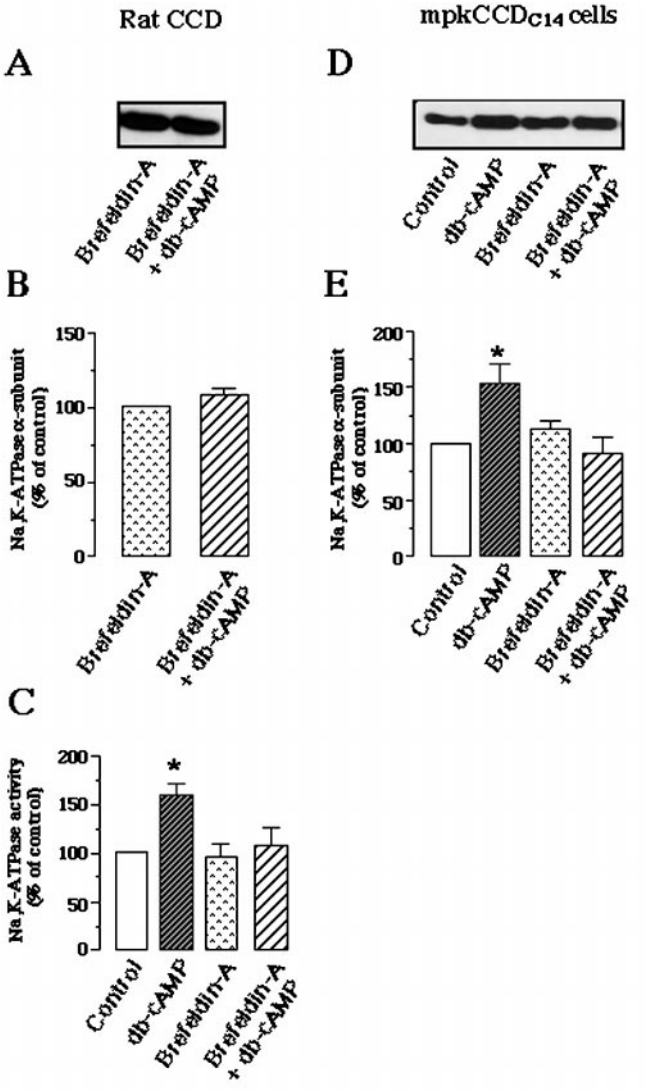

Figure 6. Brefeldin A prevented the cell surface expression and stimulation of Na,K-ATPase induced by db-cAMP. Microdissected rat CCDs or mpkCCD$_{c14}$ cells were pretreated in absence or presence of 20 μg/ml brefeldin A for 1 h at 30°C and then incubated (15 for CCDs and 30 for mpkCCD$_{c14}$ cells) with or without 10^{-3} M db-cAMP at 37°C. Tubules and cells were then biotinylated, lysed, and the labeled proteins were precipitated by streptavidin agarose-beads. The Na,K-ATPase α-subunit was detected by Western blotting as described in Figure 3. (A and D) Representative immunoblots from CCD (A) and mpkCCD$_{C14}$ cells (D), respectively. (B and E) Bars represent the densitometric quantification (means ± SE) from four independent experiments. Results (means ± SE) are expressed as percentage of the optical density values from untreated samples (control); *, $p < 0.05$ versus control values. (C) Hydrolytic activity of Na,K-ATPase in rat CCDs treated with brefeldin A and db-cAMP as described above. Values (means ± SE from 7 independent experiments) are percentage of control (370 ± 53 pmol ATP · $mm^{-1} \cdot h^{-1}$). *, $p < 0.05$ versus control values.

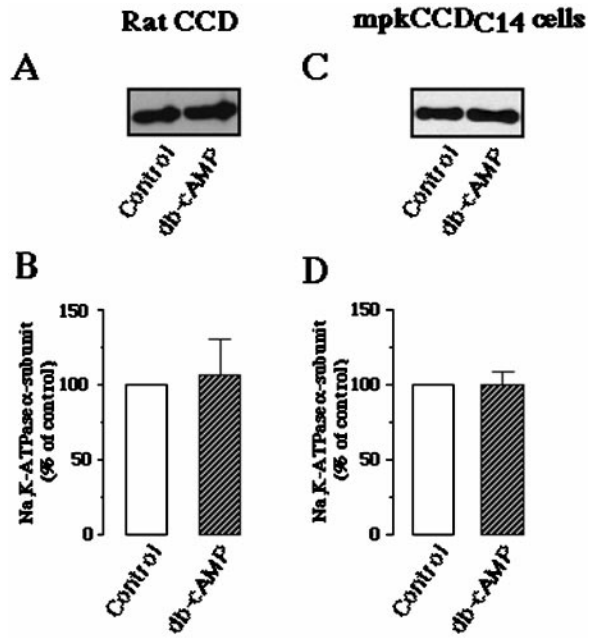

Figure 7. Low temperature (20°C) prevented the increased cell surface expression of Na,K-ATPase induced by db-cAMP. Microdissected rat CCDs or mpkCCD$_{c14}$ cells were incubated at 20°C in the absence or in the presence of 10^{-3} M db-cAMP for 30 min before biotinylation, lysis, and precipitation by streptavidin agarose-beads. Na,K-ATPase α-subunit was detected by Western blotting as described in Figure 3. (A and C) Representative immunoblots from CCD (A) and mpkCCD$_{C14}$ cells (C), respectively. (B and D) Bars represent the densitometric quantification (means ± SE) from 11 and 5 independent experiments for rat CCDs and mpkCCD$_{c14}$ cells, respectively. Results are expressed as percentage of the optical density values from untreated samples (control).

ester; Molecular Probes, Eugene, OR). Figure 7 shows that incubation of rat CCDs and mpkCCDc14 cells with 10^{-3} M db-cAMP for 30 min at 20°C did not increase the cell surface expression of Na,K-ATPase (as percentage of control; rat CCD, db-cAMP, 107 ± 23%, n = 11; NS; mpkCCD$_{c14}$ cells, db-cAMP, 100 ± 10%; n = 5; NS). As shown in Figure 8, in both rat CCDs and mpkCCD$_{c14}$ cells pretreatment with BAPTA-AM abolished the stimulation of cell surface expression of Na,K-ATPase induced by the subsequent addition of db-cAMP (as percentage of BAPTA: rat CCD, db-cAMP, 108 ± 24%; n = 3; NS; and mpkCCD$_{c14}$, db-cAMP, 109 ± 19%; n = 4; NS). Figure 8C indicates that in rat CCDs, BAPTA-AM prevented the stimulation of Na,K-ATPase activity induced by db-cAMP (as pmol ATP · $mm^{-1} \cdot h^{-1}$, n = 7; control, 370 ± 53; BAPTA, 485 ± 57, NS versus control; db-cAMP, 583 ± 66, $p < 0.05$ versus control; db-cAMP + BAPTA, 318 ± 33, NS versus control).

These results indicated that the increased cell surface expression of Na,K-ATPase and stimulation of its activity induced by db-cAMP in CCD cells were dependent on temperature and on the intracellular free calcium.

DISCUSSION

The present study provides pieces of evidence that the stimulation of Na,K-ATPase activity caused by cAMP in mammalian renal collecting ducts requires the translocation to

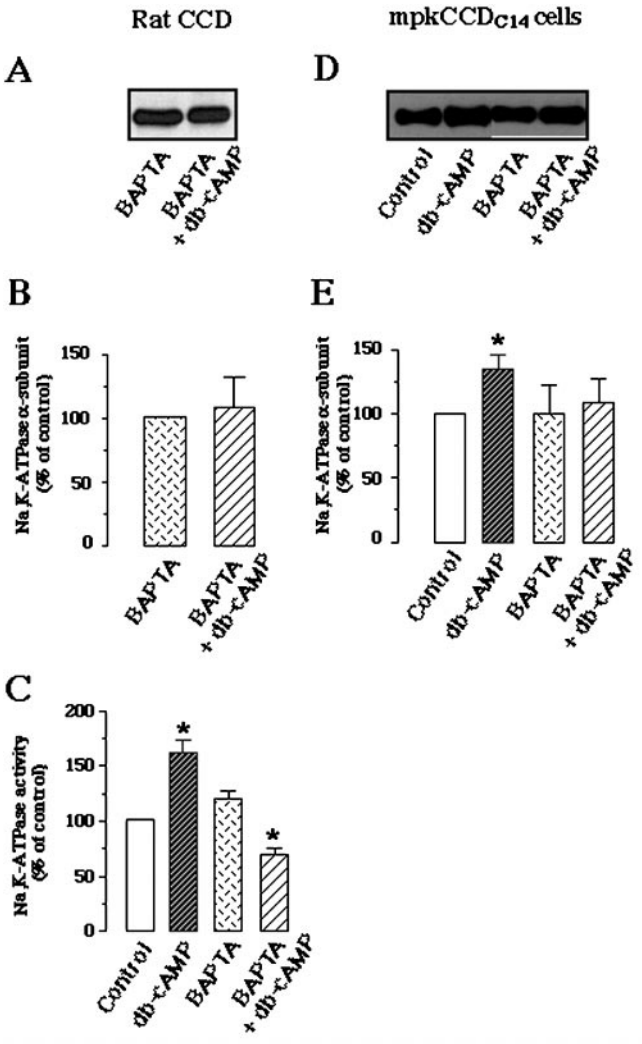

Figure 8. db-cAMP–induced cell surface expression and stimulation of Na, K-ATPase were dependent on intracellular Ca^{2+}. Microdissected rat CCDs or $mpkCCD_{c14}$ cells were preincubated without or with 10 μM BAPTA-AM at room temperature (CCDs) or at 37°C ($mpkCCD_{c14}$ cells) for 1 h, and then incubated C (15 min for CCDs and 30 min for $mpkCCD_{c14}$ cells) in the absence or presence of 10^{-3} M db-cAMP at 37°. Samples were then biotinylated, lysed, and labeled proteins were precipitated by streptavidin agarose-beads. The Na,K-ATPase α-subunit was detected by Western blotting as described in Figure 3. (A and D) Representative immunoblots from CCD (A) and $mpkCCD_{C14}$ cells (D), respectively. (B and E) Bars represent the densitometric quantification (means ± SE) from three and four independent experiments in rat CCDs and $mpkCCD_{c14}$ cells, respectively. Results (means ± SE) are expressed as percentage of the optical density values from untreated samples (control); *, $p < 0.05$ versus control values. (C) Hydrolytic activity of Na,K-ATPase in rat CCDs treated with BAPTA-AM and db-cAMP as described above. Values (means ± SE from 7 independent experiments) are percentage of control (370 ± 53 pmol ATP · mm^{-1} · h^{-1}). *, $p < 0.05$ versus control values.

the plasma membrane of an intracellular pool of Na,K-ATPase. The brefeldin A-sensitivity of this process also suggests that the fraction of Na,K-ATPase inserted in the plasma membrane in response to cAMP comes from the *trans*-Golgi network.

The stimulation of the transport activity of Na,K-ATPase by cAMP (Figure 1A) was observed in intact collecting duct in the presence of rate-limiting intracellular Na^+ and transmembrane ion gradients, i.e., a situation comparable to that prevailing in vivo (Cheval and Doucet, 1990). This is in agreement with the stimulatory effect of cAMP on Na^+ reabsorption in isolated microperfused CCDs (Schafer and Troutman, 1990; Breyer, 1991), and on epithelial Na^+ channel activity in the apical membrane of principal cells (Frindt *et al.*, 1995). Satoh *et al.* (1992) have reported an inhibition of Na,K-ATPase by cAMP in rat CCDs, but this paradoxical effect now appears to rely on an experimental bias: as demonstrated in medullary thick ascending limb of Henle (Kiroytcheva *et al.*, 1999), the phospholipase A_2- and cytochrome P450-monooxygenase–dependent synthesis of a Na,K-ATPase inhibitor induced by cAMP (Satoh *et al.*, 1992, 1993) is triggered by the inadequate metabolic and oxygen supply of the preparation.

Besides the number of active pump units located at the plasma membrane, the transport activity of tubular Na,K-ATPase is mainly determined by intracellular Na^+ concentration, by the affinity of the enzyme for Na^+ and, to a lesser extent, by basolateral K^+ conductance and membrane voltage (Féraille and Doucet, 2000). Because cAMP stimulated to the same extent (~75%) the ouabain-sensitive $^{86}Rb^+$ uptake measured under rate-limiting conditions (Figure 1A) and the Na,K-ATPase hydrolytic activity determined at V_{max} (Figure 1B), its effect appears to be independent of these regulatory factors.

In fact, the stimulatory effect of cAMP relies almost exclusively on an increase in the number of Na,K-ATPase units present in the cell membrane because the V_{max} of Na,K-ATPase activity and the amount of biotinylated α-subunits increased to the same extent (Figures 1B and 3, C and D). Similar short-term stimulation of Na,K-ATPase activity associated with increased plasma membrane density of active pumps has been documented already in skeletal muscle (Hundal *et al.*, 1992), lacrimal gland acinar cells (Gierow *et al.*, 1996), renal PCT cells (Carranza *et al.*, 1998), and lung epithelial cells (Bertorello *et al.*, 1999).

The question arises whether the observed increase in cell surface expression of Na,K-ATPase in response to cAMP resulted from an increased membrane delivery of newly synthesized Na-pump units or from the redistribution of presynthesized Na,K-ATPase units between plasma membrane and intracellular compartments. It has been suggested that vasopressin increases the synthesis of Na,K-ATPase subunits through transcriptional and translational effects in a rat collecting duct cell line (Djelidi *et al.*, 1997). However, this mechanism cannot account for the present observations because 1) the rapidity of the stimulatory effect of cAMP (10 min; Figure 4) is not compatible with a de novo synthesis and membrane insertion of new pumps, and 2) cAMP does not alter the total cellular content of Na,K-ATPase assessed by Western blotting (Figure 3, A and B) and Na,K-ATPase activity measured in saponin-permeabilized CCDs (Figure 2). These results, together with the temperature-, brefeldin A-, and calcium-sensitivity of the cAMP-induced increase in cell surface expression of Na,K-ATPase (Figures 6–8) strongly suggest that cAMP induces a redistribution of Na,K-ATPase between an intracellular and a plasma membrane pool. cAMP-induced redistribution of Na,K-ATPase between intracellular and plasma mem-

brane compartments was already reported in the rat PCT (Carranza *et al.*, 1998). It should be mentioned that the cAMP-dependent short-term redistribution of Na,K-ATPase units and long-term increase in Na,K-ATPase subunit synthesis (Djelidi *et al.*, 1997) are not mutually exclusive regulatory mechanisms.

Saponin permeabilization of CCDs allowed the measurement of the activity of the intracellular pool of Na,K-ATPase. Indeed, the effect of saponin on Na,K-ATPase activity in CCD (Figure 2) is not accounted for by a detergent effect on plasma membrane Na,K-ATPase units but rather by increasing the number of Na,K-ATPase units whose activity can be measured because 1) saponin had no stimulatory effect on vasopressin-sensitive adenylyl cyclase, another protein complex embedded in the basolateral membrane of CCD; and 2) the stimulatory effect of cAMP was no longer observed in saponin-permeabilized CCDs (Figure 2). Saponin is thought to permeabilize the membrane of cellular organelles containing the pool of Na,K-ATPase, and thereby to allow the measurement of its activity.

The 90% increase in Na,K-ATPase activity observed with saponin permeabilization compared with that obtained by the freeze/thawing method indicates that the intracellular pool of Na,K-ATPase accounts for ~50% of the total cellular pool of the enzyme. The 75% increase in cell surface expression of Na,K-ATPase in response to cAMP (Figure 3, C and D) also indicates that this intracellular pool can be mobilized almost entirely in response to a physiological stimulus. These observations imply that the Na reabsorption capacity of CCD can be modulated to a large extent according to requirements of the Na balance. In addition, results obtained with saponin-permeabilized tubules may suggest that intracellular Na,K-ATPase units are in an active state and that cAMP signaling may only redirect Na,K-pumps to the plasma membrane.

Previous studies in PCTs (Chibalin *et al.*, 1997, 1998, 1999) as well as in Cos-7 and A6 epithelial cell lines (Beron *et al.*, 1997; Féraille *et al.*, 2000) have demonstrated that Na,K-ATPase undergoes regulated endocytosis in response to dopamine or phorbols esters. The present study shows that in CCDs, cell surface Na,K-ATPase was internalized under basal condition, but that cAMP does not increase this process (Figure 5, C and D). Therefore, the cAMP-induced increase in cell surface expression of Na,K-ATPase results from an increased rate of mobilization of intracellular Na,K-ATPase units to the plasma membrane.

The sensitivity of Na,K-ATPase recruitment to brefeldin A and temperature (Figures 6 and 7) suggests that cAMP increases the delivery of Na,K-pumps, like other proteins (Muñiz *et al.*, 1996), from the *trans*-Golgi network (Traub and Kornfeld, 1997). However, one cannot rule out the involvement of specialized organelles derived from an endosomal compartment because 1) nonsecretory cells may contain a cryptic regulated secretory pathway (Chavez *et al.*, 1996); 2) sorting of proteins from early and late endosomes to synaptic-like microvesicles is sensitive to brefeldin A (Blagoveshchenskaya and Cutler, 2000); and 3) ADP ribosylation factor 1, the functional target of brefeldin A (Chardin and McCormick, 1999), is required for the recruitment of some components of the COP-I complex to early endosomes and the formation of endosomal carrier vesicles that move toward late endosomes (Gu and Gruenberg, 2000). Subcellular fractionation experiments have also shown that Na,K-ATPase is present in the early and late endosomal compartments from epithelial cells (Casciola-Rosen *et al.*, 1992; Chibalin *et al.*, 1997; Bertorello *et al.*, 1999). Furthermore, in PCT, cAMP increases Na,K-ATPase content in plasma membranes and decreases that in early endosomes (Carranza *et al.*, 1998). cAMP also increases the plasma membrane expression of Na,K-ATPase at the expense of the late endosomes in a brefeldin A-sensitive manner in lung alveolar cells (Bertorello *et al.*, 1999). Interestingly, the stimulation of the epithelial Na^+ channel in response to cAMP in epithelial cells has been shown to be brefeldin A- and temperature-sensitive (Kleyman *et al.*, 1994; Snyder, 2000). Taken together, these results suggest that similar intracellular events are involved in both apical and basolateral steps of the Na^+ reabsorption process mediated by cAMP in the collecting duct.

In rat CCD, cAMP increases intracellular Ca^{2+} (Siga *et al.*, 1994). The fact that BAPTA prevented the mobilization of Na,K-ATPase units toward the cell surface caused by cAMP (Figure 8) demonstrates the Ca^{2+} dependency of this process. However, it is not known whether BAPTA was active through preventing cAMP-induced rise in intracellular Ca^{2+} or through decreasing basal intracellular Ca^{2+} concentration. Similar Ca^{2+} dependency of cAMP stimulation of Na,K-ATPase activity has been reported in guinea pig cardiomyocytes (Gao *et al.*, 1992) and in Cos-7 cells (Cheng *et al.*, 1999), but it is not known whether cAMP induces the cell surface expression of Na,K-ATPase in these cells.

mpkCCD$_{C14}$ cells, an immortalized cell line derived from mouse collecting duct principal cells, have retained aldosterone-sensitive Na^+ transport (Bens *et al.*, 1999), and thereby represent a useful model for studying the cellular mechanism of action of mineralocorticoids in mammalian cell. The present study shows that this immortalized cell line has also retained the whole cell machinery underlying the control of Na^+ transport by cAMP, i.e., the second fundamental pathway for regulation of Na^+ balance in mammalians. Therefore, mpkCCD$_{C14}$cells represent a very powerful mammalian CCD cell system for identifying the mechanisms underlying transcriptional and posttranscriptional regulation of Na^+ transport.

In conclusion, the present study shows that cAMP rapidly mobilizes an intracellular pool of Na,K-ATPase resident in the *trans*-Golgi network toward the plasma membrane in mammalian collecting duct.

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